

Inhibition of Anaerobic Glycolysis in Ehrlich Ascites Tumor Cells by 2-Deoxy-D-Glucose*

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Little is known of the mechanisms by which 2-deoxy-D-glucose (2-DG) inhibits anaerobic glycolysis in yeast cells (1, 9, 13, 14) and in brain, diaphragm, and liver slices (15). This compound also inhibits anaerobic glycolysis in various tumors (15), prolongs the survival time of mice bearing the Krebs ascites carcinoma (2), and causes a decreased rate of growth of several tumors in rats or mice (2, 8). The inhibition with yeast (1) is competitive if either glucose or fructose serves as the substrate. 2-DG is phosphorylated by the hexokinase of yeast (1), brain (10), and tumor (15), but it is not catabolized further by these tissues. 2-DG has been claimed to inhibit the transport of monosaccharides through cell membranes (1, 9). During the course of this investigation Wick *et al.* (12) reported that 2-deoxy-D-glucose-6-phosphate (2-DGP) competitively inhibits phosphohexoisomerase in mammalian tissues.

The observation of a step preceding glycolysis and possibly involved in hexose transfer into ascites tumor cells (5, 6) prompted the study of 2-DG to characterize further the transport step in hexose utilization. The findings of Wick *et al.* (12) were confirmed for the ascites tumor, and, in addition, 2-DGP was found to inhibit glycolysis at a point subsequent to the formation of hexose diphosphate. The possibility remains, however, that 2-DG inhibits hexose transport, since the enzyme inhibitions do not fully explain the inhibitory effects on glycolysis in cells.

MATERIALS AND METHODS

The Ehrlich ascites tumor (Hauschka, clone 2 [3]) was grown in white Swiss mice. At the 8th day after inoculation with 0.2 ml. of ascites tumor, the tumor cells were collected

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in a heparinized syringe, centrifuged at $400 \times g$ for 5 minutes, and then washed twice with Krebs-Ringer bicarbonate buffer at pH 7.4. Small amounts of contaminating erythrocytes which collected at the bottom of the centrifuge tube were removed each time with a capillary pipette. After suspension to about 33 per cent (v/v) concentration in alkaline, isotonic KCl (4), the cells were homogenized either by application and rapid release of a pressure of 1500 lb/sq in of N_2 in a small stainless steel cylinder at room temperature or by 100 passes in a Brendler homogenizer at 5° C.

Anaerobic glycolysis was measured in a Warburg apparatus at 37° C. with an atmosphere of 95 per cent N_2 - 5 per cent CO_2 (11). Experiments with intact cells were performed in Krebs-Ringer bicarbonate buffer; those with homogenates in a medium modified from that of LePage (4) (Table 2). The manometric measurements were confirmed by simultaneously determining the disappearance of hexose from the medium (5, 6).¹ Tumor extracts containing both hexokinase and phosphohexoisomerase were obtained by centrifuging a homogenate at $20,000 \times g$ for 30 minutes at 5° C. A lipid layer rising to the top was discarded. The active enzymes were contained in the aqueous supernate. The activities of hexokinase and phosphohexoisomerase were measured by direct spectrophotometric assay (7) (Chart 1). The formation of reduced triphosphopyridine nucleotide (TPNH) was followed by measuring the absorption at 340 m μ in a Model DU Beckman spectrophotometer with a photomultiplier attachment. The sample of glucose-6-phosphate dehydrogenase (Sigma) used in the assay was free of hexokinase and phosphohexoisomerase activity. Doubling the amount of all reactants except the tumor enzyme did not increase the rate of the reaction, whereas doubling the amount of the tumor enzyme increased the reaction rate. The tumor extracts apparently contained phosphogluconate dehydrogenase, since the ratio, TPNH production/hexose consumption, was approximately 2.

Glucose and fructose were Pfanstiehl reagents. 2-DG was kindly supplied by Dr. Harold Blumenthal. 2-DGP was prepared by incubating 111 μ moles 2-DG with 2 mg. yeast hexokinase (Sigma), 150 μ moles disodium adenosine triphosphate (ATP) (Schwarz), and 300 μ moles $MgCl_2$ in 2.0 ml. of a 0.2 M phosphate buffer at pH 7.4. After 30 minutes of incubation at 30° C., the solution was placed in a boiling water bath for 2 minutes, and the precipitated protein was then removed by centrifugation. The solution was stored at -15° C. Fructose-6-phosphate (F-6-P) (Nutritional Biochemical Corporation) was neutralized to pH 7.0 with K_2CO_3 before use.

RESULTS

When equimolar concentrations of 2-DG and glucose were supplied to intact Ehrlich cells, a

¹ M. W. Nirenberg and J. F. Hogg, unpublished results.

reduced rate of glycolysis was observed. Under similar conditions, 2-DG completely inhibited fructolysis (Table 1). In homogenates (Table 2), when 2-DG alone was used as a substrate, CO₂ was displaced from the bicarbonate buffer, presumably as a result of the conversion of 2-DG to 2-DGP. The measurement of acid production in homogenates, then, is not a true measure of inhibition of the entire pathway of glycolysis by this compound. When the amount of CO₂ produced by the conversion of 2-DG to 2-DGP was subtracted from the CO₂ obtained with substrates plus 2-DG,

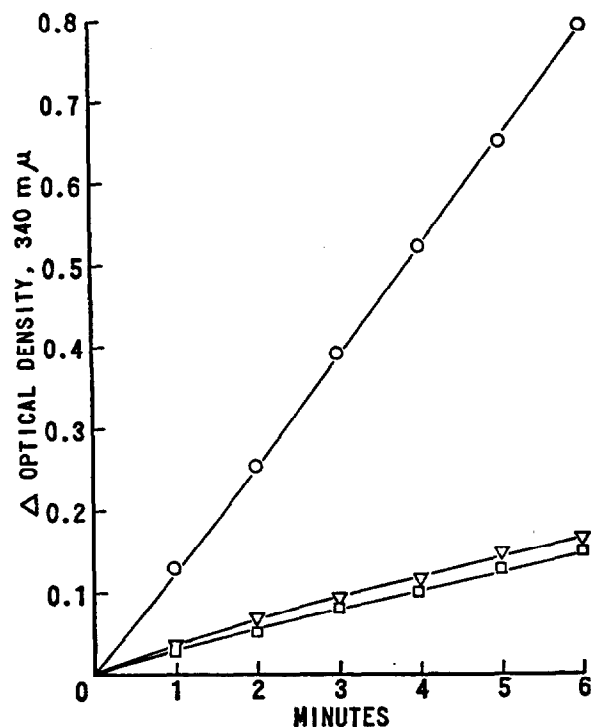


CHART 1.—The effect of 2-deoxy-D-glucose-6-phosphate upon Ehrlich ascites tumor phosphohexoisomerase. ○, 2.7 μmoles F-6-P; □, 2.7 μmoles F-6-P + 22.0 μmoles 2-DGP; ▽, 22.0 μmoles 2-DGP. The medium contained, in 3.0 ml.: appropriate substrates, 100 μmoles tris(hydroxymethyl)-aminomethane buffer (pH 8.0), 5 μmoles ATP, 10 μmoles MgCl₂, 1.2 μmoles triphosphopyridine nucleotide (TPN) (Schwarz), 5 μmoles KF, 0.2 K units of glucose-6-phosphate dehydrogenase, and sufficient tumor extract (0.05–0.10 ml.) to give the desired reaction rate.

a large inhibition of glycolysis became apparent. The maximum rate of acid production, however, was still observed with 2-DG present. Therefore, since glucose might prevent phosphorylation of 2-DG, it was possible that no inhibition had occurred in the homogenates.

To eliminate this qualification of conclusion, more specific measurements were made. First,

the effect of 2-DG upon tumor hexokinase was tested spectrophotometrically. An equimolar concentration of 2-DG had little effect upon glucose phosphorylation but had a large inhibitory effect with fructose, the change of optical density per minute being 0.11–0.12 for all combinations except fructose + 2-DG, when the rate was 0.074. These results could be explained by the possibility (a) that 2-DG competed more successfully with fruc-

TABLE 1

THE EFFECT OF 2-DEOXY-D-GLUCOSE UPON THE RATE OF ANAEROBIC GLYCOLYSIS OF EHRlich ASCITES TUMOR CELLS

Each vessel contained 2.2 ml. of a 3 per cent cell suspension which was incubated for 90 minutes. No endogenous glycolysis was observed.

| Additions per vessel (μmoles) | CO ₂ /hr/mg dry wt cells (μl.) | Per cent inhibition |
|----------------------------------|--|------------------------|
| 11 Glucose | 26.8 | |
| 11 Fructose | 21.1 | |
| 11 Glucose+11 2-DG | 19.4 | 28 |
| 11 Fructose+11 2-DG | 0 | 100 |

TABLE 2

THE EFFECT OF 2-DEOXY-D-GLUCOSE UPON THE RATE OF ANAEROBIC GLYCOLYSIS OF AN EHRlich ASCITES TUMOR HOMOGENATE

Each vessel contained 3.3 ml. of a 3 per cent tumor homogenate. CO₂ production was measured at 20-minute intervals for 80 minutes. The medium consisted of: 0.0024 M K₂HPO₄, 0.025 M KHCO₃, 0.04 M nicotinamide (Merck), 0.002 M disodium adenosine triphosphate (ATP), 0.0004 M diphosphopyridine nucleotide (DPN) (Pabst), 0.0005 M potassium fructose-1,6-diphosphate (HDP) (Schwarz), 0.0005 M pyruvate (Schwarz), and 0.01 M MgCl₂.

| Additions per vessel (μmoles) | CO ₂ /hr/mg dry wt (μl.) | CO ₂ /hr/mg dry wt Corrected for 2-DG | Apparent inhibition (per cent) |
|----------------------------------|---|---|--------------------------------------|
| None | 8.4 | | |
| 16.7 2-DG | 23.8 | | |
| 16.7 Glucose | 32.2 | | |
| 16.7 Fructose | 31.7 | | |
| 16.7 Glucose+16.7 2-DG | 34.2 | 10.4 | 68 |
| 16.7 Fructose+16.7 2-DG | 29.5 | 5.7 | 82 |

tose for hexokinase or (b) that 2-DGP inhibited the phosphohexoisomerase reaction required in the assay with fructose. The latter possibility was checked by using F-6-P as substrate in the same system, thereby permitting an assay for phosphohexoisomerase. A high level of 2-DGP completely inhibited the tumor phosphohexoisomerase (Chart 1), the inhibition being reversed by higher levels of F-6-P (Chart 2). Thus the inhibition of homogenate glycolysis by 2-DG (Table 2) was verified.

The inhibition of tumor phosphohexoisomerase

by 2-DGP could explain the inhibitory action of 2-DG upon glucose utilization by the cells, but it could not explain the even greater inhibition of fructose utilization. Fructolysis via the Embden-Meyerhof pathway, in contrast to the coupled system for the spectrophotometric assay, bypasses the phosphohexoisomerase step. This suggested that 2-DGP was also inhibiting one or more re-

added substrate. The 2-DGP inhibited the glycolysis of F-6-P, thus demonstrating inhibition of a step in the glycolytic pathway after the formation of F-6-P. Although one might expect 2-DGP to inhibit phosphofructokinase, 2-DGP again inhibited glycolysis greatly when additional HDP was supplied as substrate. Since equal inhibitions by 2-DGP were obtained with F-6-P or HDP, clearly 2-DGP can inhibit the Embden-Meyerhof pathway at some point after phosphofructokinase action.

DISCUSSION

2-DGP inhibition of HDP utilization and of phosphohexoisomerase clearly is a mode of inhibition of cellular glycolysis by 2-DG. With intact tumor cells, however, fructolysis was much more sensitive to 2-DG inhibition than glycolysis. Furthermore, fructolysis does not pass through

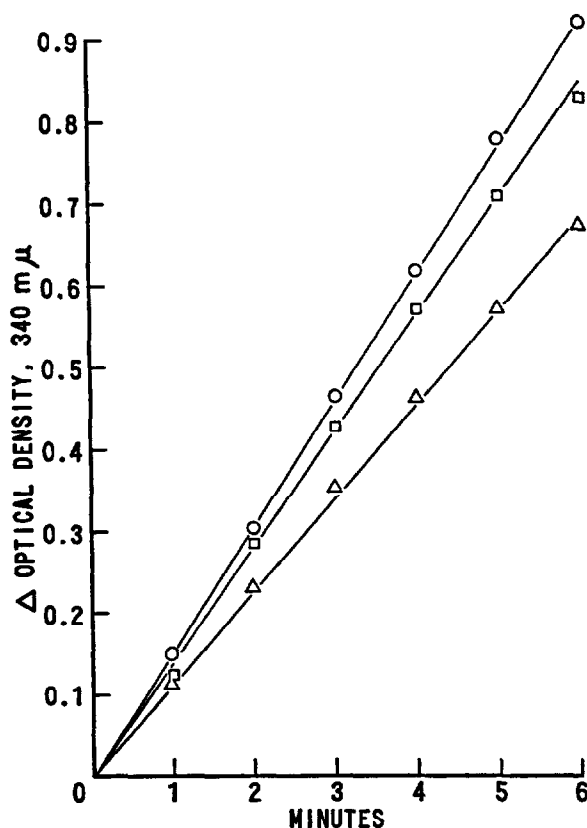


CHART 2.—Reversal of 2-deoxy-D-glucose-6-phosphate inhibition of Ehrlich ascites tumor phosphohexoisomerase by fructose-6-phosphate. ○, 2.5 μ moles F-6-P; Δ, 2.5 μ moles F-6-P + 13.9 μ moles 2-DGP; □, 20 μ moles F-6-P + 13.9 μ moles 2-DGP. See Chart 1 for medium.

actions of the Embden-Meyerhof pathway following the formation of F-6-P. This possibility was tested by adding various combinations of 2-DGP, F-6-P, and fructose-1,6-diphosphate (HDP) to tumor homogenates and determining the rate of anaerobic glycolysis manometrically (Table 3). Since the phosphorylated inhibitor was used, the previous problem of acid production from 2-DG was eliminated here. A small amount of HDP was required in the medium routinely used for homogenate experiments in order to obtain an adequate rate of glycolysis (4); therefore a low rate of glycolysis occurred in the vessels containing no

TABLE 3
THE EFFECT OF 2-DEOXY-D-GLUCOSE-6-PHOSPHATE UPON
THE RATE OF ANAEROBIC GLYCOLYSIS OF AN
EHRlich ASCITES TUMOR HOMOGENATE

See Table 2 for conditions.

| Additions per vessel (μ moles) | CO ₂ /hr/mg dry wt (μ l.) | Per cent inhibition |
|--|---|------------------------|
| None (1.5 HDP in medium) | 11.4 | |
| 22.0 2-DGP | 5.8 | 49 |
| 7.5 F-6-P | 22.6 | |
| 7.5 F-6-P + 22.0 2-DGP | 7.5 | 66 |
| 7.5 HDP | 17.0 | |
| 7.5 HDP + 22.0 2-DGP | 5.2 | 69 |

the phosphohexoisomerase step. Therefore, the action of 2-DGP cannot fully explain 2-DG inhibition of cellular glycolysis. Possibly 2-DG inhibits fructolysis by serving as a competitive substrate for hexokinase. The Michaelis-Menten constants of brain hexokinase for D-glucose, 2-DG, and D-fructose are 8.0×10^{-6} M, 2.7×10^{-5} M, and 1.6×10^{-3} M, respectively (10). Therefore, at an equimolar concentration, 2-DG should compete effectively for hexokinase with fructose, but not with glucose. Alternatively, 2-DG may compete with glucose and fructose for the hexose transfer mechanism, fructolysis being the more inhibited because fructose has the lesser affinity for the binding site (5, 6). Regardless of what may be the additional mode of inhibition by 2-DG, its use as a tool for the study of hexose transport is seriously qualified by its conversion to an effective inhibitor of glycolytic enzymes.

SUMMARY

Equimolar concentrations of 2-deoxy-D-glucose inhibited anaerobic glycolysis of Ehrlich ascites

tumor cells when either D-glucose or D-fructose was used as the substrate, glycolysis with the latter being more strongly inhibited. 2-Deoxy-D-glucose did not inhibit anaerobic glycolysis primarily at the monosaccharide transport level. Instead it was converted to 2-deoxy-D-glucose-6-phosphate by tumor hexokinase and the latter compound inhibited both phosphohexoisomerase and a further step in the Embden-Meyerhof pathway subsequent to phosphofructokinase action. The Michaelis-Menten constants of hexokinase suggest that 2-deoxy-D-glucose may also inhibit hexokinase action on D-fructose.

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